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(54) Title: BRIDGED POLYCATIONIC POLYMER-OLIGONUCLEOTIDE CONJUGATES AND METHODS FOR PREPARING SAME

(57) Abstract

$$R_2-[XR_1\underset{NH_2}{\underset{|}{C}}HCO]_a-R_3 \qquad (I)$$

$$R_2-[\underset{R_7}{\underset{|}{X}}R_6COX\underset{R_4-R_5}{\underset{|}{C}}HCO]_a-R_3 \qquad (II)$$

$$R_2-[X\underset{R_5-R_4}{\underset{|}{C}}HCO\underset{R_7}{\underset{|}{X}}R_6CO]_a-R_3 \qquad (III)$$

$$R_2-\{[NH(CH_2)_b]_c[NH(CH_2)_d]_e\}_f[NH(CH_2)_g]_h-NHR_2 \qquad (IV)$$

$$R_2-[X\underset{R_4-R_5}{\underset{|}{C}}HCO]_i-R_3 \qquad (V)$$

The present invention pertains to bridged polycationic polymer-oligonucleotide conjugates comprising a polycationic polymer covalently bonded at each end to a polyanionic oligonucleotide via a cross-linking agent, wherein the polycationic polymer may be represented by formula (I) or formula (II) or formula (III) or formula (IV) or formula (V), wherein X is -NH-, -O-, or -S-; R_1 is a lower-alkyl chain having from 1 to 4 carbon atoms; R_2 and R_3 are cross-linking agents; R_4 is a lower-alkyl chain having from 1 to 5 carbon atoms; R_5 is a primary amine, secondary amine, tertiary amine, quaternary ammonium salt, imidazole, or a guanidine group; R_6 is an alkyl chain having from 1 to 2 carbon atoms; R_7 is hydrogen, a lower-alkyl group having from 1 to 5 carbon atoms, $-CH_2C_6H_5$, or $-(CH_2)_zCOR_3$, wherein z is from 0 to 3; a is from about 3 to about 12; b is from about 2 about 5; c is from about 0 to about 3; d is from about 2 to about 5, e is from about 0 to about 3, f is from about 2 to about 9; g is from about 2 to about 5, h is from about 0 to about 3; i is from about 3 to about 12; and the ratio of cations in the polycationic polymer to anions in the polyanionic oligonucleotide pair ranges from about 1:08 to about 1.5:1, respectively.

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ISDOCID: <WO___9506056A1_I_>

BRIDGED POLYCATIONIC POLYMER-OLIGONUCLEOTIDE CONJUGATES AND METHODS FOR PREPARING SAME

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of application serial no. 07/850,555, filed 6 April 1992.

Field of the Invention

This invention pertains to bridged polycationic polymer-oligonucleotide conjugates. The bridged conjugates comprise a polycationic polymer covalently bonded at each end to a polyanionic oligonucleotide via a cross-linking agent. The polycationic polymer bridging the two polyanionic oligonucleotides helps the oligonucleotides to bind to complementary strands in a cooperative manner and aids hybridization through interaction with the oligonucleotide. The bridged conjugates have important applications in antisense and antigene fields.

Description of the Background

The disclosures referred to herein to illustrate the background of the invention and to provide additional detail with respect to its practice are

incorporated herein by reference and, for convenience, are numerically referenced in the following text and respectively grouped in the appended bibliography.

Synthetic oligonucleotides provide a new approach for controlling cellular or viral gene expression at the transcription or translation level (1, 2, 3). Oligonucleotides, however, are highly sensitive to cellular nucleases and do not effectivly pass through cellular membranes. Hence, oligonucleotides have been chemically modified in order to meet the requirements for therapeutic applications (4, 5).

One of the major ways to modify oligonucleotides is to append non-nucleic acid moieties to the oligonucleotides. Oligonucleotide-intercalator conjugates have been prepared and found to have different properties depending on the attached intercalators. Oligonucleotide-acridine conjugates have been shown to increase the binding affinity of the oligonucleotide to its complementary single-stranded target or double-stranded target (6, 7). An oligonucleotide-phenanthroline conjugate has been shown to cleave double strand DNA in the presence of cupric ion and a reducing agent (8). Cationic polylysine conjugated to oligonucleotides has been shown to improve cellular uptake, nuclease stability, and binding affinity (9).

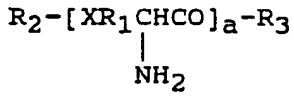
Recently, non-nucleic acid moieties were reported to link short oligonucleotides to alter hybridization properties of oligonucleotides. A pair of tethered oligonucleotide probes complementary to two noncontiguous sites on RNA was demonstrated to bind cooperatively and monomerically to the RNA target (10). The tethered bridge containing phosphodiester units had negative charges and may have a repulsive effect. A neutral poly(ethylene glycol) tether was used later to minimize electrostatic effects (11). In these cases, no interaction between the bridge and nucleic acid target was reported. Oligonucleotides linked by a terephthalamide group were found to exhibit marked enhancement in stability of duplex or triple helix formation (12). DNA triplex formation was also enhanced by bridged oligonucleotides with linker groups containing phosphate and 1,3-propanediol (oxyphosphini cooxy, 13) or hexaethylene glycol groups (14).

While a number of cationic polymers have been conjugated to oligonucleotides to improve the stability and cellular uptake of the oligonucleotides, none of these conjugated oligonucleotides have been entirely satisfactory. Conjugated cationic polymers have not been designed to substantially neutralize the oligonucleotide. The present invention provides such oligonucleotide conjugates

having improved therapeutic properties. The oligonucleotide conjugates can be synthetically modified to vary the reactivity, the specificity, and the general utility of the oligonucleotide to greatly facilitate gene regulation. The bridged conjugates of the present invention may be employed with pharmaceutically acceptable carriers to provide a wide variety of pharmaceutical products.

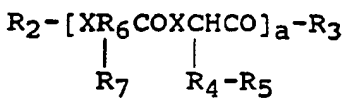
SUMMARY OF THE INVENTION

The present invention pertains to bridged polycationic polymer-oligonucleotide conjugates comprising a polycationic polymer covalently bonded at each end to a polyanionic oligonucleotide via a cross-linking agent, wherein the polycationic polymer may be represented by the formula:



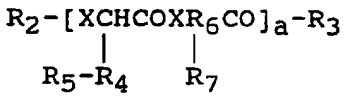
(I)

or the formula:



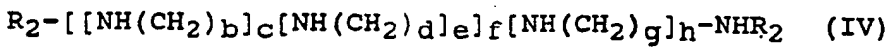
(II)

or the formula:



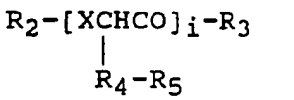
(III)

or the formula:



(IV)

or the formula:



(V)

wherein X is selected from the group consisting of -NH-, -O-, and -S-; R_1 is a substituted or unsubstituted branched or unbranched lower-alkyl chain having from 1 to 4 carbon atoms in the chain; R_2 and R_3 are cross-linking agents covalently linking the polycationic polymer to the oligonucleotide; R_4 is a substituted or unsubstituted branched or unbranched lower-alkyl chain having from 1 to 5 carbon

atoms in the chain; R_5 is selected from the group consisting of primary amines, secondary amines, tertiary amines, quaternary ammonium salts, imidazoles, and guanidine groups; R_6 is an alkyl chain having from 1 to 2 carbon atoms; R_7 is selected from the group consisting of hydrogen, branched and unbranched lower-alkyl groups having from 1 to 5 carbon atoms, $-CH_2C_6H_5$, and $-(CH_2)_zCOR_3$, wherein z is an integer ranging from 0 to 3 and R_3 is as defined above; a is an integer ranging from about 3 to about 12; b is an integer ranging from about 2 to about 5; c is an integer ranging from about 0 to about 3; d is an integer ranging from about 2 to about 5; e is an integer ranging from about 0 to about 3; f is an integer ranging from about 2 to about 9; g is an integer ranging from about 2 to about 5; h is an integer ranging from about 0 to about 3; i is an integer ranging from about 3 to about 12; and the ratio of cations in the polycationic polymer to anions in the polyanionic oligonucleotide pair ranges from about 1:8 to about 1.5:1, respectively.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a scheme illustrating the synthesis of bridged polycationic polymer-oligonucleotide conjugates.

Figure 2 is a chromatogram on a Nucleogen DEAE 60-7 column. The following abbreviations are used throughout this specification to refer to the bridged conjugates: *I* refers to the 9-mer 5'-TAA TGT GAT-3'; *II* refers to the 9-mer 5'-GAC TAG GTG-3'; R_n refers to polyarginine where n is an integer denoting the number of arginines in the polymer; and *(D-R)* refers to D-arginine. **Figure 2A** shows the purification of R_3I conjugate. **Figure 2B** shows the purification of IIR_3I bridged conjugate. **Figure 2C** shows a coinjection of five components: (1) R_3I, (2) 9-mer I, (3) iodoacetylated 9-mer I, (4) IR_3R_3I (control sample), and (5) IIR_3I.

Figure 3 is a photograph of a native 20% polyacrylamide gel after silver staining.

Figure 4 illustrates mixing curves for formation of complexes.

Figure 5 shows melting curves at 260 nm UV absorption.

Figure 6 shows the effects of peptide and gap size on T_M.

Figure 7 shows the effects of salt concentration on T_M.

DETAILED DESCRIPTION OF THE INVENTION

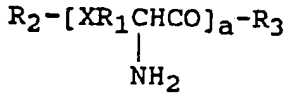
The present invention pertains to bridged polycationic polymer-oligonucleotide conjugates comprising a polycationic polymer covalently bonded at each end to a polyanionic oligonucleotide via a cross-linking agent. The cations in the polycationic polymer, such as cationic side chains in a polycationic peptide, are attached to the backbone of the polymer in a specific and repetitive manner mimicking the pattern of attachment of the anions (phosphate groups) in the nucleic acids of the polyanionic oligonucleotide. Each positively charged group in the polycationic polymer is positioned to be close to a negatively charged phosphate group in the oligonucleotide to neutralize the negative charges in the oligonucleotide through electrostatic interactions. The peptide bridge can also interact with nucleic acid targets through electrostatic interactions or hydrogen bonds thereby increasing binding affinity. Different functional groups may also be introduced into the peptide bridge to give additional properties to these compounds.

Arginine-rich motifs have been found in many nucleic acid binding proteins, such as human immunodeficiency virus (HIV) Tat protein (15), antiterminators, Gag proteins, ribosomal proteins, HIV Rev protein (16), and etc. Arginine peptides and even arginine amino acid showed specific RNA recognition (17). Therefore, peptide bridges containing arginines would be expected to have strong interaction with the phosphate groups and bases of nucleic acids. In accord with the present invention, two different 9-mers complementary to neighboring but non-contiguous sequences of single-stranded DNA target were covalently linked to both ends of a cationic peptide bridge, Cys-$(Arg)_n$-Cys (n=3, 5, 7). The C-terminal and N-terminal cysteines provided selective attachment to the two short oligonucleotides. The hybridization of bridged conjugates to complementary strands was evaluated by mixing curves, peptide effect, gap size effect and salt effect. Physicochemical studies showed that the peptide bridge helped the two 9-mers to bind to the complementary strands in a cooperative manner. Unlike single linkage peptide-oligonucleotide conjugates, the conformations of the polycationic peptides are more restricted in bridged conjugates enabling the bridged peptide to interact with the target. In addition, the increase of melting temperatures with the

addition of positive charges on the peptide suggested that the peptide bridge help the hybridization through interaction with the oligonucleotide. This type of bridged oligonucleotide conjugate may have unique properties as antisense or antigene compounds.

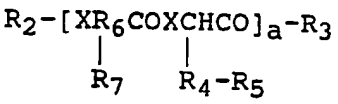
The bridged conjugates of the present invention are a new class of oligonucleotides having unique properties. For example, the bridged conjugates have an enhanced strength of hybridization of the oligonucleotides to the target nucleic acid because the ionic repulsion inherent between the two polyanionic oligonucleotide strands is reduced. Furthermore, the polycationic bridge may catalyze degradation of the target RNA strand, see Perello, M., Barbier, B., and Brack, A., "Hydrolysis of Oligoribonucleotides by *alpha*-helical Basic Peptides", Int. J. Peptide Protein Res. 38 (1991), pp. 154-160. The bridged conjugates also have enhanced stability *in vivo* because degradation is minimized when the 3'- and 5'-termini of the oligonucleotides, individually or simultaneously, are blocked by linkage to the polycationic polymer. The ability of the bridged conjugates to enter cells is also enhanced because uncharged oligonucleotides can more readily penetrate through cellular membranes. Chemotherapeutic agents such as intercalators, cell targeting agents, transmembrane delivery agents, and nucleases may also be coupled to the polycationic polymer to further enhance the activity of the bridged conjugates.

As set out above, the bridged polycationic polymer-oligonucleotide conjugates of the present invention comprise a polycationic polymer covalently bonded at each end to a polyanionic oligonucleotide via a cross-linking agent. The polycationic polymers may be represented by the formula:



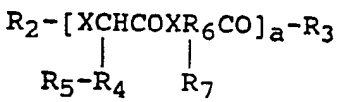
(I)

or the formula:



(II)

or the formula:



(III)

or the formula:

$$R_2\text{-}[[NH(CH_2)_b]_c[NH(CH_2)_d]_e]_f[NH(CH_2)_g]_h\text{-}NHR_2 \quad (IV)$$

or the formula:

$$R_2\text{-}[X\underset{\substack{|\\ R_4\text{-}R_5}}{C}HCO]_i\text{-}R_3 \quad (V)$$

The polyanionic oligonucleotides in the present invention may be any natural or synthetic oligonucleotide known in the art. The polyanionic oligonucleotides may be oligodeoxyribonucleic acids (normal DNA), oligoribonucleic acids (normal RNA), backbone-modified oligonucleotides such as methylated RNA and phosphorothioate oligodeoxyribonucleic acids (-OP(S)(O)O-), and combinations of normal and backbone-modified oligonucleotides. The oligonucleotide sequence may be a sense strand or an antisense strand (complementary to either a DNA or RNA sequence, *i.e.*, the sense strand, of the gene to be inhibited). Preferably, the oligonucleotide sequence is an antisense strand. Antisense carriers and backbone-modified oligonucleotides are more fully described in *Stein et al.*, *New Jersey Medicine*, *87*, pp. 1011-1015 (1990), *Stein et al.*, *Journal of Liquid Chromatography, 11*, pp. 2005-2017 (1988), and *Miller, Bio/Technology, 9*, pp. 358-362 (1991), which disclosures are incorporated herein by reference.

The oligonucleotides may be in either the 5'-->3' or the 3'-->5' orientation with respect to the polycationic polymer. The oligonucleotides may be either the same or different, and preferably are different. While the polyanionic oligonucleotides of the present invention are not limited to any specific number of nucleotide monomers, the polyanionic oligonucleotides preferably each comprise from about 5 to about 24 nucleotides, more preferably from about 6 to about 16 nucleotides, and most preferably from about 7 to about 14 nucleotides.

The polycationic polymers in the present invention may be natural or synthetic polypeptides, pseudopeptides, or polyamines. The cations in the polycationic polymer are attached to the backbone of the polymer in a specific and repetitive manner mimicking the pattern of attachment of the anions in the oligonucleotide. Preferably, the cation is selected from the group consisting of primary amines, secondary amines, tertiary amines, quaternary ammonium salts, imidazoles, and guanidine groups.

Pseudopeptides contain backbone modifications such as amide bond surrogates. The term "surrogate" as used herein refers to an amide bond modification which involves replacement of a naturally occurring amide bond by an unnatural bond. Nonlimiting examples of amide bond modifications include ketones ($-COCH_2-$), thioesters (-COS-), substituted amides (-CONR-), esters (-COO-), thioamides (-CSNH-), amines ($-CH_2NH-$), alkyl groups ($-CH_2CH_2-$), sulfides ($-CH_2S-$), sulfoxides ($-CH_2SO-$), sulfones ($-CH_2SO_2-$), alkene groups (-CH=CH-), and unnatural amide groups (-NHCO-). The term "pseudopeptide" as used herein refers to a peptide analog containing an amide backbone modification. The term "pseudopeptide" also refers to a peptide analog having amide bonds employing side chain amines instead of *alpha*-amines. Peptide backbone modifications are generally employed to confer stability to a peptide against enzymatic or proteolytic degradation such as to prepare an orally active peptide or a peptide having sustained activity. Amino acids containing amide bond modifications are termed pseudoamino acids. Peptide backbone modifications are well known in the art and are discussed in detail in "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins", Volume 7, B. Weinstein, Ed., Ch. 5, Marcel Dekker, New York, New York (1983), which disclosure is incorporated herein by reference.

In a first embodiment, the polycationic polymers are represented by the formula:

$$R_2-[XR_1\underset{\displaystyle NH_2}{\underset{|}{C}}HCO]_a-R_3 \qquad (I)$$

In Formula (I), the polycationic polymer is a pseudopeptide containing the monomer $-XR_1CH(NH_2)CO-$. Group X is a heteroatom selected from the group consisting of -NH-, -O-, and -S-. The terminal heteroatom group X on the side chain of the monomer forms amide, ester, or thioester bonds for peptide or polymer formation instead of the *alpha*-amine group. The *alpha*-amine group in the monomer is used as the repeating cationic group to form ion pairs with the anionic groups in the oligonucleotide. Preferably, group X is -NH-.

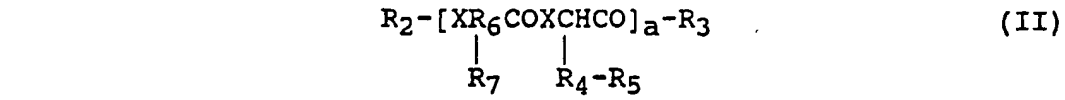
Group R_1 is a lower-alkyl spacer group which may be varied to provide the desired number of carbon atoms in the skeleton of the repeating monomer. Group R_1 is a substituted or unsubstituted branched or unbranched

lower-alkyl chain having from 1 to 4 carbon atoms in the chain. Preferably, group R_1 contains from 2 to 3 carbon atoms in the chain.

Groups R_2 and R_3 are terminal end capping groups covalently bonded to the terminal nitrogen, oxygen, or sulfur group in the polycationic polymer of Formula (I) and are cross-linking agents covalently linking the polycationic polymer to the oligonucleotide. Group R_2 will generally contain a carbonyl group for coupling R_2 to group X via an amide, ester, or thioester bond. For example, R_2 may be an acyl group having from 1 to about 15 carbon atoms, preferably from 1 to about 10 carbon atoms, and more preferably from 1 to about 6 carbon atoms. Group R_3 will generally contain an amine, hydroxyl, or sulfhydryl group for coupling R_3 to the carbonyl group of the polycationic polymer of Formula (I) via an amide, ester, or thioester bond and a hydroxyl or sulfhydryl group to form an ester or thioester bond with the phosphate group on the oligonucleotide.

The cross-linking agents which may be employed in the present invention are those agents which will covalently link the polycationic polymer to the oligonucleotide without interfering with the ion-pairing of the polycationic polymer and the oligonucleotide. Groups R_2 and R_3 may be a single cross-linking agent such as glycine or cysteine, and preferably is cysteine. Groups R_2 and R_3 may also be a combination of compounds to form a cross-linking agent such as aminolink agents, succinate cross-linking agents, cysteine, and maleimide. Nonlimiting examples of aminolink agents include 1,6-hexanolamine, and 2,3-dihydroxy-1-aminopropane. Aminolink agents contain a hydroxyl group at one end of a chain to form an ester bond with the phosphate group on the oligonucleotide and an amine group at the other end of the chain to form an amide bond with a carbonyl group. Nonlimiting examples of succinate cross-linking agents include those agents derived from ethylene glyco*bis*(sulfo succinimidylsuccinate) (Sulfo-EGS), and maleimidobenzoyl-sulfosuccinimide ester (sulfo-MBS). Cysteine may also be coupled to the succinate cross-linking agent and then further coupled to the polycationic polymer via the terminal amine or carbonyl group. In one embodiment, the combination of cross-linking agents is an aminolink agent coupled to the oligonucleotide and further coupled to a succinate cross-linking agent, the succinate cross-linking agent is in turn coupled to a cysteine, which is in turn coupled by its carbonyl group to group X in the polycationic polymer of Formula (I).

In a second embodiment, the polycationic polymers are represented by the formula:



$$R_2\text{-}[XR_6(R_7)COXCH(R_4\text{-}R_5)CO]_a\text{-}R_3 \qquad \text{(II)}$$

In Formula (II), the polycationic polymer is a polypeptide or pseudopeptide containing the monomer $-XR_6(R_7)COXCH(R_4\text{-}R_5)CO-$ which comprises two different types of amino acids or pseudo amino acids. Groups X, R_2, and R_3 are defined as set out above. A first amino acid or pseudoamino acid ($-XCH(R_4\text{-}R_5)CO-$) contains a side chain ($-R_4-$) having a cationic group ($-R_5$), such as an amine group, imidazole group, or a guanidine group, which is used as the repeating cationic group to form ion pairs with the oligonucleotide. The *alpha*-amine group or group X in this first amino acid forms the amide, ester, or thioester bonds for peptide or polymer formation. A second amino acid or pseudoamino acid ($-XR_6(R_7)CO-$) is coupled to the first amino acid and is used as a spacer group to provide the desired skeleton size of the repeating monomer unit. R_7 may be a functionalized side chain for attaching additional groups to the polycationic polymer such as cross-linking agents and chemotherapeutic agents.

Group R_4 is a lower-alkyl side chain group to which cationic group R_5 is coupled. The length of the side chain may be varied to provide the desired number of carbon atoms in the chain. Group R_4 is a substituted or unsubstituted branched or unbranched lower-alkyl chain having from 1 to 5 carbon atoms in the chain. Preferably, group R_4 contains from 2 to 4 carbon atoms in the chain.

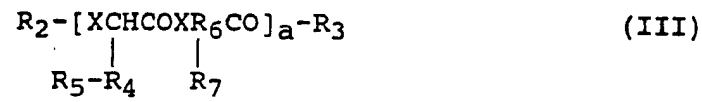
Group R_5 is a cationic group attached to lower-alkyl side chain group R_4. The type of cationic group employed in R_5 may be varied to optimize the ion-pairing properties with the particular oligonucleotide. Group R_5 is selected from the group consisting of primary amines, secondary amines, tertiary amines, quaternary ammonium salts, imidazoles, and guanidine groups ($-NHC(=NH)NH_2$). Preferably, group R_5 is selected from the group consisting of primary amines, secondary amines, and guanidine groups ($-NHC(=NH)NH_2$). More preferably, group R_5 is a guanidine group ($-NHC(=NH)NH_2$).

Group R_6 is a lower-alkyl spacer group which may be varied to provide the desired number of carbon atoms in the skeleton of the repeating

monomer. Group R_6 is an alkyl chain having from 1 to 2 carbon atoms in the chain. Preferably, group R_6 contains 1 carbon atom in the chain.

Group R_7 may be hydrogen; a side chain such as those found in alanine, leucine, isoleucine, valine, or phenylalanine; or a functionalized side chain. When group R_6 contains two carbon atoms, group R_7 may be attached to either carbon atom. Group R_7 is selected from the group consisting of hydrogen, branched and unbranched lower-alkyl groups having from 1 to 5 carbon atoms, $-CH_2C_6H_5$, and $-(CH_2)_zCOR_3$. Preferably, the branched and unbranched lower-alkyl groups have from 1 to 4 carbon atoms, more preferably from 1 to 3 carbon atoms. The integer z may range from 0 to 3, preferably from 0 to 2. Group R_3 is as defined above. When R_7 contains a functionalized side chain such as an acyl group
($-(CH_2)_zCOR_3$), additional groups (R_3 groups) may be attached to the polycationic polymer such as cross-linking agents and chemotherapeutic agents. Preferably, group R_7 is selected from the group consisting of hydrogen and branched and unbranched lower-alkyl groups having from 1 to 5 carbon atoms. More preferably, group R_7 is selected from the group consisting of hydrogen and branched and unbranched lower-alkyl groups having from 1 to 3 carbon atoms. Most preferably, group R_7 is hydrogen.

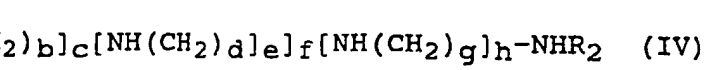
In a third embodiment, the polycationic polymers are represented by the formula:



$$R_2\text{-}[XCHCOXR_6CO]_a\text{-}R_3 \qquad \text{(III)}$$
$$\quad\;\; |\qquad\;\; |$$
$$\quad R_5\text{-}R_4 \quad R_7$$

In Formula (III), the polycationic polymer is a variation of the polycationic polymer set out above in Formula (II). The polycationic polymer in Formula (III) is a polypeptide or pseudopeptide containing the monomer $-XCH(R_4\text{-}R_5)COXR_6(R_7)CO-$, wherein the C-terminal and N-terminal positions of the two different types of amino acids or pseudo amino acids set out above in Formula (II) are reversed. Groups X and R_2 through R_7 are defined as set out above.

In a fourth embodiment, the polycationic polymers are represented by the formula:



$$R_2\text{-}[[NH(CH_2)_b]_c[NH(CH_2)_d]_e]_f[NH(CH_2)_g]_h\text{-}NHR_2 \qquad \text{(IV)}$$

In Formula (IV), the polycationic polymer is a polyamine containing the monomer -$NH(CH_2)_b$- or the monomer -$NH(CH_2)_d$-, or both. The monomer -$NH(CH_2)_g$- permits the polycationic polymers of Formula (IV) to contain an odd number of monomers. Each group R_2 may be the same or different and is defined as set out above. The secondary amine groups in the polyamines are an integral part of the skeleton of the polycationic polymer and are used as the repeating cationic group to form ion pairs with the oligonucleotide. In general, these polyamines are derived by hydride reduction of the amide bonds in the corresponding polypeptides to give polyamines of the spermidine and spermine type. For example, the peptide *beta*-alanine-*gamma*-aminobutyric acid-*beta*-alanine-carboxy amide yields spermine upon reduction. Different sequences of these amino acids or other combinations of amino acids, including cysteine, may be used to generate any desired polyamine. Alternatively, commercially available polyamines may be suitably derivatized with the R_2 groups.

In a fifth embodiment, the polycationic polymers are represented by the formula:

$$R_2-[XCHCO]_i-R_3 \qquad (V)$$
$$\quad\;\; |$$
$$\quad R_4-R_5$$

In Formula (V), the polycationic polymer is a peptide containing the monomer -$XCH(R_4-R_5)CO$-. Groups X and R_2 through R_5 are defined as set out above. In this embodiment, the monomer -$XCH(R_4-R_5)CO$- is preferably arginine and R_2 and R_3 are cysteine.

The integer a may range from about 3 to about 12, preferably from about 3 to about 8, and more preferably from about 4 to about 7. The integer b may range from about 2 to about 5, preferably from about 2 to about 4, and more preferably from about 3 to about 4. The integer c may range from about 0 to about 3, preferably from about 1 to about 3, and more preferably from about 2 to about 3. The integer d may range from about 2 to about 5, preferably from about 2 to about 4, and more preferably from about 3 to about 4. The integer e may range from about 0 to about 3, preferably from about 1 to about 3, and more preferably from about 2 to about 3. The integer f may range from about 2 to about 9, preferably from about 2 to about 5, and more preferably from about 2 to about 3. The integer g may range from about 2 to about 5, preferably from about 2 to about

4, and more preferably from about 3 to about 4. The integer h may range from about 0 to about 3, preferably from about 1 to about 3, and more preferably from about 2 to about 3. The integer i may range from about 3 to about 12, preferably from about 3 to about 7, more preferably from about 5 to about 7, and most preferably 7.

In general, the ratio of cations in the polycationic polymer to anions in the polyanionic oligonucleotide pair will range from about 1:8 to about 1.5:1, preferably from about 1:6 to about 1:2, and more preferably from about 1:4 to about 1:3, respectively. Bridged conjugates containing an excess of cations may have useful properties such as enhanced uptake of the bridged conjugate into cells or into the nucleus of cells or may have ribonuclease-like activity, Perello, M., Barbier, B., and Brack, A., "Hydrolysis of Oligoribonucleotides by *alpha*-helical Basic Peptides", Int. J. Peptide Protein Res. 38 (1991), pp. 154-160.

The term "branched lower-alkyl chain" means a lower-alkyl chain or skeleton to which is attached branching lower-alkyl groups having from about 1 to about 4 carbon atoms, preferably from about 1 to about 3 carbon atoms. The substituent groups attached to the lower-alkyl chain or branching lower-alkyl groups may be selected from the group consisting of hydroxyl, sulfhydryl, halogen, and lower-alkoxy groups having from 1 to 4 carbon atoms. The term halogen, as used herein, refers to the chemically related elements consisting of fluorine, chlorine, bromine, and iodine.

The bridged conjugates of the present invention have high specificity because the sequence of each particular antisense oligonucleotide can be selected to be complementary to a specific target. The bridged conjugates also have high affinity hybridization because the ion-pairing properties of the polycationic polymer chain lowers the ionic repulsion between the two polyanionic oligonucleotides, as demonstrated by analogy with methylphosphonate oligonucleotides, R. S. Quartin and J. G. Wetmur, "Effect of Ionic Strength on the Hybridization of Oligodeoxynucleotides With Reduced Charge Due to Methylphosphonate Linkages to Unmodified Oligodeoxynucleotides Containing the Complementary Sequence", Biochemistry 28 (1989) pp. 1040-1047, and as shown by R. Eritja, A. Pons, M. Escareller, E. Giralt, and F. Albericio, "Synthesis of Defined Peptide-Oligonucleotide Hybrids Containing a Nuclear Transport Signal Sequence", Tetrahedron 47 (1991), pp. 4113-4120. Because hybridization strength (*i.e.*, T_m or melting temperature) is dependent upon the length and sequence of an oligonucleotide, shorter antisense chains of the strong affinity bridged conjugates of

the present invention may be sufficient for hybridizing a particular oligonucleotide with a target nucleic acid.

The bridged conjugates also have enhanced stability *in vivo* because exonuclease digestion is minimized when both the 3'- and 5'-termini are blocked such as by linking the polycationic polymers of the present invention to the oligonucleotides. Similarly, polypeptide degradation is minimized when both the amino-terminus and carboxy-terminus are blocked by linkage to the oligonucleotide, thereby limiting exopeptidase digestion. Endopeptidase digestion is also minimized when pseudopeptide bonds are employed in the polypeptide.

The bridged conjugates have enhanced bioavailability because the ability of the oligonucleotides to penetrate through cellular membranes is enhanced when the negative charges on the antisense DNA are ion-paired, by analogy to methylphosphonate and other non-ionic antisense oligonucleotides, P. S. Miller, K. B. McParland, K. Jayaraman, and P. O. P. Ts'o, "Biochemical and Biological Effects of Nonionic Nucleic Acid Methylphosphonates", Biochemistry 20 (1981), pp. 1874-1880. Additional hydrophobic groups such as valine, phenylalanine, intercalators, and other groups may be coupled to, or incorporated in, the polycation polymer to enhance permeability.

Alternatively, an excess of cations in the oligonucleotide-conjugate can be used to enhance permeability. Enhanced oligonucleotide uptake occurs through binding of the positively charged paired-ion oligonucleotide to the negatively charged outer membrane of the cell followed by endocytosis, P. L. Schell, "Uptake of Polynucleotides by Mammalian Cells XIV: Stimulation of the Uptake of Polynucleotides by Poly(L-lysine", Biochim. Biophys. Acta 340, (1974) pp. 323-333). A combination of hydrophobic and positively charged groups may be synergistic with regard to the ability of the antisense substance to reach the necessary compartments *in vivo*, such as the nuclei of cells, R. Eritja, A. Pons, M. Escareller, E. Giralt, and F. Albericio, "Synthesis of Defined Peptide-Oligonucleotide Hybrids Containing a Nuclear Transport Signal Sequence", Tetrahedron 47 (1991), pp. 4113-4120.

The bridged conjugates have low toxicity because the metabolic degradation products of polypeptide conjugates are amino acids and nucleotides. Concern has been expressed about the toxicity of phosphorothioate and other backbone-modified DNA-analogs, S. Agrawal, J. Goodchild, M. P. Civeira, A. H. Thornton, P. S. Sarin, and P. C. Zamecnik, "Oligodeoxynucleoside

Phosphoramidates and Phosphorothioates as Inhibitors of Human Immunodeficiency Virus", Proc. Natl. Acad. Sci. USA 85, (1988) pp. 7079-7083; Y. Cheng, W. Gao, and F. Han, "Phosphorothioate Oligonucleotides as Potential Antiviral Compounds Against Human Immunodeficiency Virus and Herpes Viruses", Nucleosides & Nucleotides 10 (1991), pp. 155-166. Increasing the potency of the antisense oligonucleotide should result in a better therapeutic index.

The cost per dose of the bridged conjugates should be reasonable especially for a high potency product because, in the preferred size range of 5-25 nucleotide monomers, oligodeoxyribonucleotides and polypeptides are routinely synthesized on automated instruments.

Figure 1 is a scheme illustrating the synthesis of bridged polycationic polymer-oligonucleotide conjugates. The following abbreviations are used throughout this specification to refer to the bridged conjugates: *I* refers to the 9-mer 5'-TAA TGT GAT-3'; *II* refers to the 9-mer 5'-GAC TAG GTG-3'; R_n refers to polyarginine where n is an integer denoting the number of arginines in the polymer; and *(D-R)* refers to D-arginine.

Figure 2 is a chromatogram on a Nucleogen DEAE 60-7 column. **Figure 2A** shows the purification of *R3I* conjugate. **Figure 2B** shows the purification of *IIR3I* bridged conjugate. **Figure 2C** shows a coinjection of five components: (1) *R3I*, (2) 9-mer I, (3) iodoacetylated 9-mer I, (4) *IR3R3I*, and (5) *IIR3I*. Chromatographic conditions are described in Table 1. Detection was at UV 260 nm.

Figure 3 is a photograph of a native 20% polyacrylamide gel after silver staining. Lane 1: 9-mer I, Lane 2: *R3I*, Lane 3: *R5I*, Lane 4: *R7I*, Lane 5: *IIR3I*, Lane 6: *IIR5I*, Lane 7: *IIR7I*, Lane 8: *II(D-R)3I*, Lane 9: *II(D-R)5I*, Lane 10: C18-mer (the seqence is the combination of 9-mer I and 9-mer II, 5'-TAA TGT GAT GAC TAG GTG-3').

Figure 4 illustrates mixing curves for formation of complexes. (O) Mixing of 9-mer I plus 9-mer II with T24-mer. (♦) Mixing of *IIR3I* with T24-mer. Fraction X presents mole fraction of T24-mer.

Figure 5 shows melting curves at 260 nm UV absorption.

		T_M(°C)
1.	9-mer I plus 9-mer II, T24-mer, gap=3,	25.0, 34.0
2.	*R3I* plus 9-mer II, T24-mer, gap=3,	36.0
3.	*IIR7I* bridged conjugate, T24-mer, gap=3,	51.0
4.	*IIR5I* bridged conjugate, T24-mer, gap=3,	49.5
5.	*IIR3I* bridged conjugate, T24-mer, gap=3,	48.5

Figure 6 shows the effects of peptide and gap size on T_M.

		T_M(°C)
1.	*IIR3I* bridged conjugate, T18-mer, gap=0,	46.5
	bridged conjugate, T24-mer, gap=3,	48.5
	bridged conjugate, T25-mer, gap=4,	48.0
	bridged conjugate, T27-mer, gap=6,	46.5
2.	*IIR5I* bridged conjugate, T18-mer, gap=0,	47.5
	bridged conjugate, T24-mer, gap=3,	49.5
	bridged conjugate, T25-mer, gap=4,	49.5
	bridged conjugate, T27-mer, gap=6,	48.5
3.	*IIR7I* bridged conjugate, T18-mer, gap=0,	49.0
	bridged conjugate, T24-mer, gap=3,	51.0
	bridged conjugate, T25-mer, gap=4,	51.0
	bridged conjugate, T27-mer, gap=6,	50.0

Figure 7 shows the effects of salt concentration on T_M. Duplex of *IIR3l* and T18-mer (gap=0), or T24-mer (gap=3), or T25-mer (gap=4), or T27-mer (gap=6). 1. No NaCl, 2. 0.1 M NaCl, 3. 1.0 M NaCl.

In another embodiment, the present invention is directed to paired-ion oligonucleotides comprising a polyanionic oligonucleotide covalently bonded to a polycationic polymer via a cross-linking agent, wherein the polycationic polymer is represented by the formula:

$$R_2-[\underset{\displaystyle R_4-R_5}{\underset{|}{XCHCO}}]_i-R_3 \qquad (V)$$

wherein Group X and integer i is defined as set out above; R_2 is independently selected from the group consisting of hydrogen, acyl groups, cross-linking agents covalently linking the polycationic polymer to the oligonucleotide, and chemotherapeutic agents; R_3 is selected from the group consisting of -NRR', -OR'', cross-linking agents covalently linking the polycationic polymer to the oligonucleotide, and chemotherapeutic agents; R_4 is a substituted or unsubstituted branched or unbranched lower-alkyl chain having from 1 to 5 carbon atoms in the chain; R_5 is selected from the group consisting of primary amines, secondary amines, tertiary amines, quaternary ammonium salts, imidazoles, and guanidine groups; R and R' may be the same or different and are independently selected from the group consisting of hydrogen and branched and unbranched lower-alkyl groups, and R'' is selected from the group consisting of branched and unbranched lower-alkyl groups, each lower-alkyl group having from 1 to 6 carbon atoms; wherein at least one of R_2 or R_3 is a cross-linking agent; and the ratio of cations in the polycationic polymer to anions in the polyanionic oligonucleotide ranges from about 8:10 to about 16:8, respectively.

In a preferred embodiment of the paired-ion oligonucleotides, the oligonucleotide is an antisense oligonucleotide; X is -NH-; R_2 is selected from the group consisting of cross-linking agents and chemotherapeutic agents; R_3 is selected from the group consisting of -NRR', cross-linking agents, and chemotherapeutic agents; R_4 has from 2 to 4 carbon atoms in the chain; R_5 is selected from the group consisting of primary amines and secondary amines; the cross-linking agent comprises an aminolink agent, a succinate cross-linking agent, cysteine, and maleimide; the chemotherapeutic agent is selected from the group consisting of

intercalators, cell targeting agents, transmembrane delivery agents, natural and synthetic nucleases, and free radical generators; and the ratio of cations in the polycationic polymer to anions in the polyanionic oligonucleotide ranges from about 9:10 to about 10:9, respectively.

In yet another embodiment, the present invention is directed to a pharmaceutical composition comprising a pharmaceutically acceptable carrier and the paired-ion oligonucleotide set out above.

In still yet another embodiment, the present invention is directed to a method for preparing the paired-ion oligonucleotide comprising a polyanionic oligonucleotide covalently bonded to a polycationic polymer via a cross-linking agent, which comprises the steps of:

(1) providing the polyanionic oligonucleotide;

(2) providing the polycationic polymer;

(3) coupling the polyanionic oligonucleotide to the polycationic polymer via the cross linking agent.

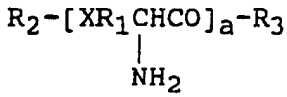
The present invention extends to methods for preparing the bridged conjugates. The bridged conjugates may be synthesized using standard techniques and apparatus known to those skilled in the art. The ultimate bridged conjugates are readily prepared using methods generally known in the chemical and biochemical arts.

In general, the polycationic peptides can be synthesized by standard peptide chemistry coupling reactions such as by N-Fmoc [N-(9-fluorenyl-methoxycarbonyl] chemistry on a peptide synthesizer. In one embodiment, the amino acids may be coupled to a PAL™ support by BOP [benzotriazolyl-N-oxytris(dimethylamino) phosphonium hexafluorophosphate] and HOBt (1-hydroxybenzotriazole). The side chain protecting groups may be removed by TFA (trifluoroacetic acid) at the time when the peptide is cleaved from the solid support. The peptide product may be purified by reverse-phase HPLC and the identity of the product may be confirmed by amino acid analysis, peptide sequencing, and mass spectrometry.

A sense or antisense oligonucleotide having a primary amine group coupled through its 5'- or 3'-termini, or through an internal position, may be synthesized on an automated DNA synthesizer using commercially available reagents. After HPLC purification and removal of the protecting groups, the

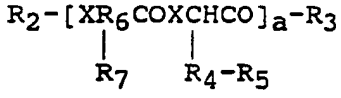
oligonucleotide may be coupled to the polycationic polymer via a cross-linking agent such as a succinimide-mediated chemical reaction. The polycationic polymer and oligonucleotide conjugate may then be deprotected and purified by HPLC or on an anion-exchange column.

In a specific embodiment, the invention is directed at a method for preparing bridged polycationic polymer-oligonucleotide conjugates comprising a polycationic polymer covalently bonded at each end to a polyanionic oligonucleotide via a cross-linking agent, wherein the polycationic polymer may be represented by the formula:



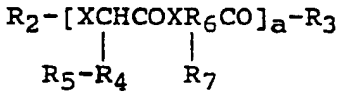
(I)

or the formula:



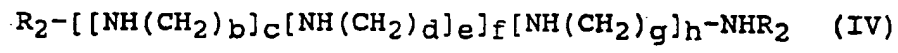
(II)

or the formula:



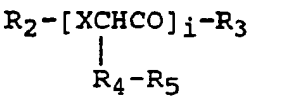
(III)

or the formula:



(IV)

or the formula:



(V)

wherein X is selected from the group consisting of -NH-, -O-, and -S-; R_1 is a substituted or unsubstituted branched or unbranched lower-alkyl chain having from 1 to 4 carbon atoms in the chain; R_2 and R_3 are cross-linking agents covalently linking the polycationic polymer to the oligonucleotide; R_4 is a substituted or unsubstituted branched or unbranched lower-alkyl chain having from 1 to 5 carbon atoms in the chain; R_5 is selected from the group consisting of primary amines, secondary amines, tertiary amines, quaternary ammonium salts, imidazoles, and guanidine groups; R_6 is an alkyl chain having from 1 to 2 carbon atoms; R_7 is selected from the group consisting of hydrogen, branched and unbranched lower-

alkyl groups having from 1 to 5 carbon atoms, $-CH_2C_6H_5$, and $-(CH_2)_zCOR_3$, wherein z is an integer ranging from 0 to 3 and R_3 is as defined above; a is an integer ranging from about 3 to about 12; b is an integer ranging from about 2 to about 5; c is an integer ranging from about 0 to about 3; d is an integer ranging from about 2 to about 5; e is an integer ranging from about 0 to about 3; f is an integer ranging from about 2 to about 9; g is an integer ranging from about 2 to about 5; h is an integer ranging from about 0 to about 3; i is an integer ranging from about 3 to about 12; and the ratio of cations in the polycationic polymer to anions in the polyanionic oligonucleotide pair ranges from about 1:8 to about 1.5:1, respectively;

which comprises the steps of:

(1) providing the polyanionic oligonucleotides;

(2) providing the polycationic polymer;

(3) coupling the polyanionic oligonucleotides to the polycationic polymer via the cross linking agents.

The bridged conjugates may be prepared using standard techniques and equipment known to those skilled in the art. The apparatus useful in accordance with the present invention comprises apparatus well known in the chemical and biochemical arts, and therefore the selection of the specific apparatus will be apparent to the artisan.

The bridged conjugates of the present invention may be used together with pharmaceutically acceptable carriers to provide pharmaceutical compositions which can be administered to mammals such as man in amounts effective to provide a variety of therapeutic activity. Suitable carriers include propylene glycol-alcohol-water, isotonic water, sterile water for injection (USP), emulphor™-alcohol-water, cremophor-EL™ or other suitable carriers known to those skilled in the art. Other suitable carriers include isotonic water, sterile water for injection (USP), alone or in combination with other solubilizing agents such as ethanol, propylene glycol, or other conventional solubilizing agents known to those skilled in the art.

Of course, the type of carrier will vary depending upon the mode of administration desired for the pharmaceutical composition as is conventional in the art. A preferred carrier is an isotonic aqueous solution of the inventive compound.

The compounds of the present invention can be administered to mammals, e.g., animals or humans, in amounts effective to provide the desired therapeutic effect. Since the activity of the compounds and the degree of the desired therapeutic effect vary, the dosage level of the compound employed will also vary. The actual dosage administered will also be determined by such generally recognized factors as the body weight of the patient and the individual hypersensitiveness of the particular patient. Thus, the unit dosage for a particular patient (human) can be as low as about 0.001 mg/kg, or about 0.1 mg in a 100 kg person, which the practitioner may titrate to the desired effect.

The compounds of the present invention can be administered parenterally, in the form of sterile solutions or suspensions, such as intravenously, intramuscularly or subcutaneously in the carriers previously described.

For parental therapeutic administration, the compounds of the present invention may be incorporated into a sterile solution or suspension. These preparations should contain at least about 0.1% of the inventive compound, by weight, but this amount may be varied to between about 0.1% and about 50% of the inventive compound, by weight of the parental composition. The exact amount of the inventive compound present in such compositions is such that a suitable dosage level will be obtained. Preferred compositions and preparations according to the present invention are prepared so that a paranteral dosage unit contains from between about 0.1 milligrams to about 100 milligrams of the inventive compound.

The sterile solutions or suspensions may also include the following adjuvants: a sterile diluent, such as water for injection, saline solution, fixed oils, polyethylene glycol, glycerine, propylene glycol, or other synthetic solvent; antibacterial agents, such as benzyl alcohol or methyl paraben; antioxidants, such as ascorbic acid or sodium metabisulfite; chelating agents, such as ethylenediaminetetraacetic acid (EDTA); buffers, such as acetates, citrates or phosphates; and agents for the adjustment of tonicity, such as sodium chloride or dextrose. The parental preparations may be enclosed in ampules, disposable syringes, or multiple dose vials made of glass or plastic.

It is especially advantageous to formulate the pharmaceutical compositions in dosage unit forms for ease of administration and uniformity of dosage. The term dosage unit forms as used herein refers to physically discrete units suitable for use as a unitary dosage, each unit containing a predetermined

quantity of active ingredient calculated to produce the desired therapeutic effect in association with the pharmaceutical carrier.

The present invention is further illustrated by the following examples which are not intended to limit the effective scope of the claims. All parts and percentages in the examples and throughout the specification and claims are by weight of the final composition unless otherwise specified.

Examples

Materials and Methods

Trifluoroacetic acid (TFA) and 5'-amino-modifier-C6-TFA were from Applied Biosystems (Foster city, CA). Iodoacetic acid, tributylphosphine (Bu_3P), anisole, ethanedithiol, and lithium chloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Fmoc-L-Cys(StBu)-OH was from Bachem California (Torrance, CA). Acetic Acid, sodium chloride, sodium hydroxide, sodium acetate, and sodium bicarbonate were from EM Science (Gibbstown, NJ). ISS oligo staining system was from Integrated Separation Systems (Natick, MA). Acetonitrile and methylene chloride from J. T. Baker, triethylamine, ethyl ether were purchased from Fisher Scientific (Springfield, NJ). 3'-amino-modifier C7 CPG was from Glen Research (Sterling, VA). Fmoc-L-Cys(Trt)-OH/BOP+HOBt, and Fmoc-Arg(Pmc)-OH/BOP+HOBt cartridges (0.5 mmole), PAL™ support (0.1 mmole), HOBt and BOP reagent were from Milligen/Biosearch (Milford, MA). N-hydroxysuccinimide (NHS), 6N hydrochloric acid, and dimethyl sulfoxide were from Pierce Chemical Co. (Rockford, IL). 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), and ethylenediaminetetraacetic acid (EDTA) were from Sigma Chemical Co. (St. Louis, MO). 3-(N-morpholino) propanesulfonic acid (MOPS) and glycerol were from United States Biochemical Corp. (Cleveland, OH).

Preparation of H-Cys(StBu)-$(Arg)_n$-Cys-NH_2

The peptides, having a free amino-terminus but an amidated carboxy-terminus, were synthesized in a 9400 Excell Peptide Synthesizer (Milligen/Biosearch, Burlington, MA) using the Fmoc chemistry on a 0.1 mmole scale. Prepacked amino acid cartridges were used directly, except Fmoc-L-Cys(StBu)-OH (215.8 mg, 0.5 mmole), which was packed with BOP reagent (221.3 mg, 0.5 mmole) and HOBt (67.5 mg, 0.5 mmole) before use. After the

synthesis, the peptide was cleaved with 5 ml of TFA/anisole/ethanedithiol (95/4/1, v/v), precipitated from ethyl ether, and purified by reverse-phase HPLC on a Vydac (Separation group, Hesperia, CA) 218TP1022, 10 mm, C-18 column (2.2 x 25 cm) using a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 4 ml/min. The structure of the purified peptide was verified by fast atom bombardment mass spectroscopy (FAB). Ellman assay with DTNB (18) showed 0.9 mole free thiol group per mole peptide, based on peptide weight.

Preparation of Oligonucleotides

The oligonucleotides were synthesized by phosphoramidite methodology using a Model 380B DNA Synthesizer (Applied Biosystem, Foster City, CA) on a 1 mmole scale. The 9-mers were coupled to amino linkers at either the 5' or 3' terminus on the synthesizer. The 9-mer with a 5'-amino linker (9-mer I) was purified by anion-exchange chromatography on a Nucleogen DEAE 60-7 column (4 x 125 mm) (Nest Group, Southborough, MA) (19). The product was desalted by reverse-phase HPLC on a Hamilton PRP-1, 10 mm, C-18 column (4.1 x 150 mm) (VWR Scientific, Piscataway, NJ) using a gradient of acetonitrile with aqueous 0.1M triethylammonium acetate, pH 7.0 at a flow rate of 1 ml/min. Trityl-on and Trityl-off two-step purifications (20) on the PRP-1 column were done for all other oligonucleotides using a similar acetonitrile gradient.

Synthesis of Iodoacetylated Oligonucleotides

Iodoacetic acid and N-hydroxysuccinimide were used to synthesize N-[(iodoacetyl)oxy]succinimide (21). Oligonucleotide 9-mer I (5 units, A260) in 100 ml of 0.1M $NaHCO_3$ and 2 mg of N-[(iodoacetyl)oxy]-succinimide (150 eq) in 100 ml of dimethyl sulfoxide were mixed and reacted in the dark at room temperature for 2 hours. The iodoacetylated 9-mer I was purified by anion-exchange chromatography on a Nucleogen DEAE 60-7 column. Mobile phase A was 60% 20 mM sodium acetate (pH 7.0), 40% acetonitrile. Mobile phase B was mobile phase A containing 0.7M LiCl. The flow rate was 1 ml/min. The gradient was 100% A for 5 minutes, 100% A to 88% A for 20 minutes, 88% A to 50% A in 1 minute. The eluent at 35 minutes was collected, put under vacuum for 20 minutes to evaporate acetonitrile and was then ready for conjugation to the peptide.

Iodoacetylated 9-mer II was prepared in a similar manner, except that $NaHCO_3$ was added to a final concentration of 0.1M after acetonitrile was removed under vacuum.

Synthesis of Oligonucleotide-peptide Conjugates

The iodoacetylated 9-mer I in eluent buffer was allowed to react with 1 mg of H-Cys(StBu)-$(Arg)_n$-Cys-NH_2 (25 eq) dissolved in 50 ml of water under nitrogen in the dark at room temperature overnight. The reaction mixture was purified on a Nucleogen DEAE 60-7 column (Figure 2, A). The chromatogram showed a new major peak (9-mer I-peptide conjugate, Table 1), as well as small peaks at 29 minutes (unreacted 9-mer I) and 30 minutes (unreacted iodoacetylated 9-mer I). The unreacted peptide eluted with the solvent front at 1.5 minutes, as detected by UV absorption and DTNB reagent. The new major peak was collected, reduced in volume to 200 ml under vacuum, and ready for conjugation to the 9-mer II. In another preparation, the 9-mer I-peptide was also desalted on the PRP-1 column. The peak at 31 minutes was collected and dried, giving a 60% yield (3 units, A260).

Synthesis of Oligonucleotide-peptide-oligonucleotide Conjugates

The 9-mer I-peptide conjugate (3 units, A260) in 200 ml of concentrated eluent buffer was adjusted to pH 8.3 (0.1M $NaHCO_3$), was stirred vigorously with 10 ml of Bu_3P (1300 eq) (22) in 200 ml of methylene chloride under nitrogen at room temperature for 4 hours to cleave the t-butylthiol protecting group on cysteine. The iodoacetylated 9-mer II (3 units, A260, 1 eq) in 800 ml of eluent buffer and 0.1M $NaHCO_3$ was added, and the reaction mixture was stirred under nitrogen in the dark at room temperature overnight. The aqueous layer was then separated and washed with 0.5 ml of methylene chloride four times. The aqueous phase was resolved by ion-exchange chromatography, as above (Figure 2. B). The peptide-9mer I peak, 9-mer II peak (29 minutes) and iodoacetylated 9-mer II peak (30 minutes) were all small. A new later peak was collected, dried, and desalted on the PRP-1 column. The peak at 25-26 minutes was dried, giving a 50% yield (3 units, A260). The bridged conjugate *IR3I1* was made similarly as above.

Control Experiments

The bridged conjugate *IR3I* was synthesized for comparison using the same method as above. The disulfide-linked dimer *IR_3R_3I* was made similarly, but with the following modifications. After removing the *t*-butylthiol group from cysteine, the aqueous phase was washed with methylene chloride, and stirred overnight. The purification step was the same as that for bridged conjugates. A

peak at 32 minutes was observed on the Nucleogen DEAE 60-7 column. When peptide-9-mer I conjugate, without removing t-butylthiol group, was stirred with a second iodoacetylated 9-mer overnight, no reaction happened, as observed by ion-exchange chromatography.

Polyacrylamide Gel Electrophoresis (PAGE)

The conjugates was analyzed on a native 20% polyacrylamide gel. The running buffer was 40 mM MOPS and 10 mM sodium acetate (pH 7.0, adjusted with sodium hydroxide). The samples were suspended in 20 ml of loading buffer (30% glycerol), heated to 90°C for 2 minutes, chilled on ice and loaded onto the gel. Other lanes were loaded with 20 ml of 30% glycerol, 0.05% xylene, and 0.05% bromophenol blue to indicate running distance. The bands were visualized by silver staining with ISS oligo staining system kit. The picture was taken with Polaroid film (Figure 3).

Amino Acid Analysis

The samples were hydrolyzed with 5 μl of 6N HCl in hermetically sealed microcapillary tubes at 150°C for 90 minutes (23). Amino acid analysis was based on reverse phase separation on a Chromoga Bond MC-18, 3 mm column (4.6x100 mm), followed by o-phthaldialdehyde (OPA) postcolumn derivatization (24).

Mixing Curves

Stock solutions of 3 mM 9-mer I plus 9-mer II, *IIR3I*, and T24-mer were prepared in 10 mM sodium phosphate (pH 7.0), 0.1M NaCl, 0.1M EDTA. The mixing curves of duplex formation were measured similarly as described (25). The 9-mer I plus 9-mer II, *IIR3I* stock solutions was added separately to the T24-mer solution to construct the duplex formation. After each addition, the cuvettes were equilibrated at 15°C for 20 minutes, and then the absorption readings at 260 nm were recorded. In another set of data, the T24-mer solution was added to the 9-mer I plus 9-mer II, *IIR3I* stock solutions.

Melting Temperature Studies

The melting studies were done on a computer-interfaced Perkin-Elmer spectrophotometer Coleman 575 (Norwalk, CT) at 260 nm UV absorption. The samples were 3 mM of each oligomer in 1 ml of buffer composed of 10 mM sodium phosphate (pH 7.0), 0.1M NaCl, and 0.1 mM EDTA. After the samples were annealed by cooling from 90°C to 5°C at 0.5°C/min, the T_M curves were measured at 260 nm using a temperature gradient from 5°C to 90°C at 0.5°C/min.

Table 1

Names of compounds and their retention times

Compounds	Name	RT*(min)	RT**(min)
Oligonucleotides			
5'-TAA TGT GAT-3' with 5' amino linker	9-mer I	29	21
5'-GAC TAG GTG-3' with 3' amino linker	9-mer II	29	21
acetylated 9-mer I, 9-mer II		30	25-26
Single Linkage Conjugates			
Cys-(L-Arg)3-Cys-9-mer I	R3I	25	
Cys-(L-Arg)5-Cys-9-mer I	R5I	23	31
Cys-(L-Arg)7-Cys-9-mer I	R7I	21	
Bridged Conjugates			
9-mer II-Cys-(L-Arg)3-Cys-9-mer I	IIR3I	35	
9-mer II-Cys-(L-Arg)5-Cys-9-mer I	IIR5I	33	
9-mer II-Cys-(L-Arg)7-Cys-9-mer I	IIR7I	32	
9-mer II-Cys-L-Arg-D-Arg-L-Arg-Cys-9-mer I	IIR3I	35	25-26
9-mer II-Cys-(L-Arg-D-Arg)2-L-Arg-Cys-9-mer I	IIR5I	33	
9-mer I-Cys-(L-Arg)3-Cys-9-mer II	IR3II	35	
9-mer I-Cys-(L-Arg)3-Cys-Cys-(L-Arg)3-Cys-9-mer	IIR3R3I	32	

* Retention time on a Nucleogen deae 60-7 column. Buffer A: 20 mM sodium acetate (pH 7.0), 40% acetonitrile. Buffer B: buffer A plus 0.7 M LiCl. Gradiant: 100% A for 5 minutes, 100% A to 60% A in 40 minutes. Flow rate: 1 ml/min.

** Retention time on a Hamilton PRP-1 column. Buffer A: 95% 0.1 M triethylammonium acetate (pH 8.0), 5% acetonitrile. Buffer B: 5% 0.1 M

triethylammonium acetate (pH 8.0), 95% acetonitrile. Gradient: 100% A for 5 minutes, 100% A to 80% A for 40 minutes. Flow rate: 1 ml/min.

Table 2. Oligonucleotide targets used for physicochemical studies. The highlight regions present the binding sizes of 9-mer I and 9-mer II. The gap size means the number of nucleotides between two 9-mer binding sites on oligonucleotide targets.

Table 2

Sequences (5'→3')	Name	Gap Size
ATC ACA TTA CAC CAT GTC	T18-mer	0
ATC ACA TTA CTA CAC CAT GTC GTA	T24-mer	3
ATC ACA TTA CTTA CAC CAT GTC GTA	T25-mer	4
ATC ACA TTA CTT TTA CAC CAT GTC GTA	T27-mer	6

Results

Synthesis of Bridged Conjugates

In the synthesis scheme, two cysteines with two different protecting groups, trityl (cleavable by TFA) and t-butylthiol (cleavable by Bu_3P), were utilized to control the selective attachment of oligonucleotides to the peptide (Figure 1). Each peptide was deprotected in TFA/anisole/ethanedithiol (95:4:1). In the absence of 1% ethanedithiol scavenger, the desired product cannot be obtained. Titration of the collected product with DTNB reagent (Ellman assay) showed no free thiol group in the product, which indicated the modification of the thiol group upon deprotection of the trityl group. The synthesis of peptide-9-mer I conjugate was performed at neutral pH solution in order to prevent the loss of the t-butylthiol protecting group. However, basic condition (0.1M $NaHCO_3$) was found to be necessary for high yields of bridged conjugates.

All conjugation reactions were performed in salt (LiCl) solution. Because peptide-9-mer I conjugate has low solubility in water due to strong interaction of arginine side chains and phosphate groups. In salt solution, such interaction is weakened, making the conjugate readily dissolved. The bridged

conjugate can be readily dissolved in water because of a large excess of phosphate groups compared to arginine.

A sequential one-pot method was applied to the synthesis of bridged conjugates. The peptide in the conjugate was reduced first. Subsequently, the alkylating agent was added to the reaction bath in the presence of excess reducing agent (26, 27). It was found to be necessary to allow the initial reduction step to go to completion, since iodoacetic acid and their amides react with Bu_3P to form a quarternary phosphinium salt, which is much less reactive than Bu_3P.

The reducing reagent Bu_3P has the advantages over the widely used DTT in this application. First, DTT must be removed after reduction of the disulfide bond, which requires an additional separation step. Second, a substantial amount of the disulfide-linked dimer side product, 9-mer I-peptide-peptide-9-mer I was found to be formed in the DTT procedure. The presence of Bu_3P during this conjugation prevented the undesirable dimer formation, thereby increasing the yield of the final product. A large excess of Bu_3P was used, since it is readily oxidized by air. Due to the limited aqueous solubility of Bu_3P, a two-phase reaction was chosen, and the organic phase containing most of the Bu_3P was removed after the reaction was completed.

Chromatographic Purifications

The purification of conjugates was successfully achieved by anion-exchange chromatography on a Nucleogen DEAE 60-7 column (Figure 1). The separations are based on the interaction of the negative charges on short oligonucleotides with the positive charges on column packing. The fractionation of the mixture components is governed by the difference in their net charges. Organic solvent in the eluent (40% acetonitrile) minimized nonionic interactions between the oligonucleotides and packing materials. Figure 2 shows purification chromatogram of *IIR3I* conjugate. The retention time of the 9-mer with an amino linker was 29 minutes. After iodoacetylation, the new molecule eluted 1 minute later, at 30 minutes. This was possibly due to the larger size of the molecule or the elimination of one positive charge on the primary amino group by amide bond formation. After conjugation to the peptide containing three arginine residues, which had 4 positive charges, the *R3I* (with an overall charge of -5) eluted much earlier, at 25 minutes (Figure 2. A). The *IIR3I* bridged conjugate, comprised of two 9-mers and one peptide (with an overall charge of -14), eluted at 35 minutes (Figure 2. B). The IR_3R_3I disulfide-linked dimer, comprised of two 9-mers and two peptides (with

an overall charge of -10) eluted at 32 minutes. Thus, the retention times of the conjugates are proportional to their negative charges (Figure 2. C).

The retention times of all the compounds are included in Table 1. The single linkage conjugates *R3I*, *R5I*, and *R7I* eluted at 25 minutes, 23 minutes, and 21 minutes, respectively. The bridged conjugates *IIR3I* (*II(D-R)3I*), *IIR5I* (*II(D-R)5I*), and *IIR7I* eluted at 35 minutes, 33 minutes, and 32 minutes, respectively. The relative elution times are consistent with the overall charge of each compound. All the products are well resolved from the reactants.

The conjugates were desalted on a Hamilton PRP-1 column using TEAA buffer system. Certain separations can also be achieved on this reverse-phase column. But purification of single linkage conjugates from reaction mixtures cannot be performed by reverse-phase chromatography due to serious precipitation of the conjugates in the column in the presence of positive charge peptides. In anion-exchange column, this problem is solved by peptide elution with solvent front. The bridged conjugates have almost same retention times as acetylated 9-mers, therefore cannot be purified on reverse-phase column, either.

Gel Electrophoresis

Gel electrophoresis method enables separation of macromolecules according to characteristic features such as electric charge, size, shape, and secondary structure. These parameters can affect electrophoretic properties either separately or jointly. Polyacrylamide gel electrophoresis was used to identify the conjugates and check their purity (Figure 3). Each conjugate gave only one band on the gel, confirming their purity.

The electronic charge of macromolecules could be changed by the pH of the medium. Here, neutral pH was choosen to maintain the charges on the oligonucleotides and the peptides. The peptide-9-mer I conjugate migrated much more slowly than 9-mer I, due to the influence of the positive charges on the peptide. The positions of peptide-9-mer I conjugates were dependent on their charges. *R3I* had two less positive charges than *R5I* and four less than *R7I*, and migrated faster than *R5I* and *R7I*. *R7I* had only one net negative charges, and barely moved in the gel, and stayed at the top of the gel. The bridged conjugates ran fast than peptide-9-mer I, and had the same order of electrophoretic mobility as single linkage conjugates. Although the bridged conjugate has a larger size, the additional negative charges have a greater influence on migration. *IIR3I* and *II(D-*

R)3I, *IIR5I* and *II(D-R)5I* are at the same positions. Compared with C18-mer, the bridged conjugates had slower mobility because of the positive charges from the peptides. The disulfide-linked dimer *IR3R3I*, had an even slower mobility than *R3I*, due to molecular size considerations rather than charge density (data not shown). In conclusion, the electrophoretic migration order is:

9-mer > C18-mer > bridged conjugates >
single linkage conjugates > disulfide-linked dimer
(for each peptide).

Here, gel electrophoresis is a powerful tool to distinguish these compounds.

Amino Acid Analysis

The amount of each oligonucleotide conjugate was determined by optical density absorption at 260 nm. The peptide-9-mer I and the disulfide-linked dimer, 9 mer-I-peptide-peptide-9 mer I, should give 1:1 molar ratio of DNA to peptide, whereas the bridged conjugates should give 2:1 ratio of DNA to peptide. The measured values were 0.9, 0.9 and 1.9, respectively, within experimental error of the expected integer ratios.

Mixing Curves

The stoichiometry of the duplex formation of bridged conjugate and single-stranded DNA was studied by the method of continuous fractions (25). The comparison between 9-mer I:T24-mer:9-mer II di-duplex and *IIR3I*:T24-mer duplex was made. Inflection points at a mole fraction of 0.48, 0.47 were obtained respectively for 9-mer I:T24-mer:9-mer II di-duplex and *IIR3I*:T24-mer duplex from the mixing curves (Figure 4). 9-mer I and 9-mer II bind to two different sites of T24-mer. The mixing curves proved that once two 9-mers were linked by a peptide bridge, they are still able to bind cooperatively to the target by Watson-Crick duplex formation.

Melting Curves

The hybridization of the conjugates and the parent oligonucleotides to T24-mer having complementary sequences was determined by analysis of UV absorbance versus temperature melting curves (Figure 5). The 9-mer I plus 9-mer

II had two binding sites on T24-mer, and gave two thermal transitions on melting curve 1 (T_M of 25.0°C and 34.0°C). This is because the stability of a DNA duplex depends on its base sequence (28). 9-mer II had 55% GC content and both 5' and 3' end were terminated by GC, whereas 9-mer I only contained 22% GC content and thus had a lower T_M than 9-mer II. After 9-mer I was conjugated to a peptide (as *R3I*), only one thermal transition was observed (curve 2, T_M of 36°C). The T_M increase suggested that the positive charged peptide help the hybridization of 9-mer I to the binding site of T24-mer. In both cases, no cooperative binding of 9-mer I and 9-mer II was detected. Melting curve 1 and 2 were broad, possibly due to the secondary structure of T24-mer target. The bridged conjugate, 9-mer II-peptide-9-mer I, had much higher T_M (curve 3, 4, 5). The transition curves were also considerably steeper. These results indicate that the peptide bridge allows the two 9-mers to bind to the complementary strand in a cooperative manner. Besides, melting temperatures of bridged conjugates to T24-mer were increased with the addition of the positive charges on the peptide bridges, Cys-$(Arg)_n$-Cys (n = 3, 5, 7). *IIR5I*:T24-mer duplex had a T_M of 1°C higher than *IIR3I*:T24-mer duplex. *IIR7I*:T24-mer duplex had a even higher T_M, which was 2.5°C higher than *IIR3I*:T24-mer. This evidence supports the design that the peptide bridge increases the duplex stability through the interaction with the phosphate groups and bases of the oligonucleotides. Thus, at physiological temperature (37°C), neither 9-mer would be expected to hybridize to the target, whereas strong hybridization would occur when these two 9-mers are bridged. The proper orientation of guanidine group in the peptide is very important to peptide-nucleic acid interaction. *II(D-R)3I* and *IR3II*, *II(D-R)5I* were made to see if stereochemistry have any effects on hybridization property, but they showed the same melting temperatures as *IIR3I*, *IIR5I* respectively (data not shown).

Effects of Gap Size and Peptide on Hybridization

The hybridization properties of bridged conjugates were further investigated by varying the number of nucleotides between two 9-mer binding sites on the oligonucleotide target. To minimize the influence of the sequence specificity, the sequences of two 9-mer binding sites are remained the same for all the targets. Gap size 4 and 6 were generated by adding T nucleotides to T24-mer in the gap region. The sizes of the peptide and the gap are all critical for hybridization property of a bridged conjugate. For a certain peptide bridge, a small gap would make it bulge out and cannot fit well in the gap region. But the too large gap makes the gap region bulge out and generates the difficulty for better hybridization. As in Figure 6, melting temperatures with gap size 0, 3, 4, 6 on the

target were measured. Almost the same T_M was observed with gap size 3 and 4, a T_M decrease of 2°C with gap size 0, and a decrease of 1 or 2°C with gap size 6. This means that proper space on the target helps the hybridization, and too small or too large space decrease the duplex stability. Compared three bridged conjugates tested, there was a tendency that bridged conjugate with longer peptide bridge had less decrease in T_M with the increase of the gap size. *IIR7I* bridge conjugate had stronger binding strength for all complementary strands tested than *IIR3I* and *IIR5I* conjugates. A perfect matched C18-mer:T18-mer duplex in the same condition had a T_M of 57°C (data not shown). For bridged conjugate:DNA target, the reason of the reduction in duplex stability is lack of the nearest-neighbor interaction for the last nucleotides at 3' end of 9-mer I and 5' end of 9-mer II (28).

Salt Dependence of Hybridization

To find the optimal conditions for duplex formation of bridged conjugate and DNA target, the salt dependence of hybridization was evaluated (Figure 7). The melting curves were measured at three different salt concentrations (0, 0.1M and 1.0M). A large increase in T_M (13°C) was observed when salt concentration was changed from 0 to 0.1M. There was only a 8°C increase in T_M from 0.1M to 1.0M NaCl concentration. The gap sizes did not influence this T_M change. For the perfect matched C18-mer:T18-mer, the increase was even higher from 41.5°C (no NaCl) to 57°C (0.1M NaCl), 68°C (1M NaCl), thus the two stage increases were 15.5°C and 11°C respectively. Therefore, the bridged conjugates showed less salt dependence than normal oligonucleotides.

Other Examples

Bridged conjugates using other polycations were also prepared. The bridging compounds were Cys-$(His)_3$-Cys, Cys-$(delta\text{-}Orn)_{10}$-Cys, and Cys-$(Leu\text{-}Lys)_2$-Lys-$(Leu\text{-}Lys)_2$-Cys, where the cysteine residues were utilized for linkage to the oligonucleotide pair. These compounds were synthesized, purified, and analyzed by PAGE in a manner similar to that described above.

Discussion

A procedure for synthesizing and purifying the bridged conjugates containing positive charged peptide as the bridge was designed and developed. The bridge concept can function to improve strength of hybridization through electrostatic interaction and hydrogen bonding, as proved by T_M measurements.

The gap size on the target should be taken into consideration to achieve optimal hybridization. The peptide bridge can bring other properties to oligonucleotides, such as the incorporation of a ribonuclease mimic into the bridge (29). The oligonucleotides provides strong affinity and high specificity to the substrate, acting as binding site, and the peptide mimic cleaves the recognized site of nucleic acid and then releases the cleaved fragments, acting as active site. The bridged conjugates differ from the single linkage conjugates in restricting the conformation change of the conjugated component. The synthetic pathway can be applied to synthesize the conjugates bridged by a peptide, pseudopeptide, or polyamine containing one or more positive charges. Similarly, the conjugates with negative charged bridge can also be achieved this way, since there is a change in net charges of the compounds. This provides the possibility of incorporating a variety of components as the bridge.

The hybridization property of the bridged conjugates to single-stranded DNA target was studied. The T24-mer sequence is from a fragment (base 65-88) on m-transcript of killer virus of the yeast Saccharomyces cerevisiae. This will allow the same bridged conjugates to be used in the physicochemical studies for the *in vitro* bioassay. Single-stranded RNA might be a better target for the conjugates with an arginine peptide bridge, since arginine was demonstrated to have specific RNA recognition (30). A strategic region on the target RNA may contain a specific arginine-binding motif and two single stranded complementary sequences. The bridged conjugates are possibly capable of binding alternate strands of duplex DNA to form triple helix under physiological conditions (31, 32).

Throughout this application, various publications have been referenced. The disclosures in these publications are incorporated herein by reference in order to more fully describe the state of the art.

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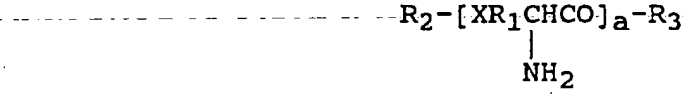
While the invention has been particularly described in terms of specific embodiments, those skilled in the art will understand in view of the present disclosure that numerous variations and modifications upon the invention are now enabled, which variations and modifications are not to be regarded as a departure from the spirit and scope of the invention. Accordingly, the invention is to be broadly construed and limited only by the scope and spirit of the following claims.

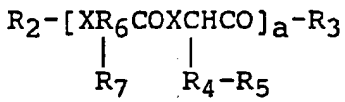
We claim:

1. A bridged polycationic polymer-oligonucleotide conjugate comprising a polycationic polymer covalently bonded at each end to a polyanionic oligonucleotide via a cross-linking agent, wherein the polycationic polymer may be represented by the formula:



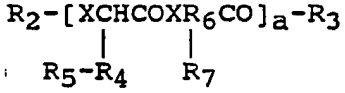
(I)

or the formula:



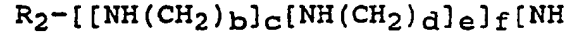
(II)

or the formula:



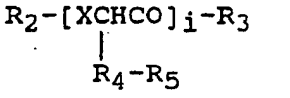
(III)

or the formula:



(IV)

or the formula:



(V)

wherein X is selected from the group consisting of -NH-, -O-, and -S-; R_1 is a substituted or unsubstituted branched or unbranched lower-alkyl chain having from 1 to 4 carbon atoms in the chain; R_2 and R_3 are cross-linking agents covalently linking the polycationic polymer to the oligonucleotide; R_4 is a substituted or unsubstituted branched or unbranched lower-alkyl chain having from 1 to 5 carbon atoms in the chain; R_5 is selected from the group consisting of primary amines, secondary amines, tertiary amines, quaternary ammonium salts, imidazoles, and guanidine groups; R_6 is an alkyl chain having from 1 to 2 carbon atoms; R_7 is selected from the group consisting of hydrogen, branched and unbranched lower-alkyl groups having from 1 to 5 carbon atoms, $-CH_2C_6H_5$, and $-(CH_2)_zCOR_3$, wherein z is an integer ranging from 0 to 3 and R_3 is as defined above; a is an integer ranging from about 3 to about 12; b is an integer ranging from about 2 to about 5; c is an integer ranging from about 0 to about 3; d is an integer ranging

from about 2 to about 5; e is an integer ranging from about 0 to about 3; f is an integer ranging from about 2 to about 9; g is an integer ranging from about 2 to about 5; h is an integer ranging from about 0 to about 3; i is an integer ranging from about 3 to about 12; and the ratio of cations in the polycationic polymer to anions in the polyanionic oligonucleotide pair ranges from about 1:8 to about 1.5:1, respectively.

2. The bridged conjugate according to claim 1, wherein each oligonucleotide independently comprises from about 5 to about 24 nucleotides.

3. The bridged conjugate according to claim 2, wherein each oligonucleotide independently comprises from about 6 to about 16 nucleotides.

4. The bridged conjugate according to claim 1, wherein X is -NH-.

5. The bridged conjugate according to claim 1, wherein R_1 has from 2 to 3 carbon atoms in the chain.

6. The bridged conjugate according to claim 1, wherein R_2 and R_3 are cysteines.

7. The bridged conjugate according to claim 1, wherein R_4 has from 2 to 4 carbon atoms in the chain.

8. The bridged conjugate according to claim 1, wherein group R_5 is guanidine.

9. The bridged conjugate according to claim 1, wherein group R_6 has 1 carbon atom.

10. The bridged conjugate according to claim 1, wherein group R_7 is selected from the group consisting of hydrogen and branched and unbranched lower-alkyl groups having from 1 to 5 carbon atoms.

11. The bridged conjugate according to claim 1, wherein a is an integer ranging from about 3 to about 8, b is an integer ranging from about 2 to about 4, c is an integer ranging from about 1 to about 3, d is an integer ranging from about 2 to about 4, e is an integer ranging from about 1 to about 3, f is an integer ranging from about 2 to about 5, g is an integer ranging from about 2 to

about 4, h is an integer ranging from about 1 to about 3, and i is an integer ranging from about 3 to about 7.

12. The bridged conjugate according to claim 1, wherein the ratio of cations in the polycationic polymer to anions in the polyanionic oligonucleotide pair ranges from about 1:6 to about 1:2, respectively.

13. The bridged conjugate according to claim 1, wherein the polycationic polymer may be represented by the formula:

Cys-$(Arg)_7$-Cys

14. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the bridged conjugate according to claim 1.

15. A method for preparing a bridged polycationic polymer-oligonucleotide conjugate comprising a polycationic polymer covalently bonded at each end to a polyanionic oligonucleotide via a cross-linking agent, wherein the polycationic polymer may be represented by the formula:

$$R_2\text{-}[XR_1\underset{\displaystyle NH_2}{\underset{|}{CH}}CO]_a\text{-}R_3 \qquad (I)$$

or the formula:

$$R_2\text{-}[X\underset{\displaystyle R_7}{\underset{|}{R_6}}COX\underset{\displaystyle R_4\text{-}R_5}{\underset{|}{CH}}CO]_a\text{-}R_3 \qquad (II)$$

or the formula:

$$R_2\text{-}[X\underset{\displaystyle R_5\text{-}R_4}{\underset{|}{CH}}COX\underset{\displaystyle R_7}{\underset{|}{R_6}}CO]_a\text{-}R_3 \qquad (III)$$

or the formula:

$$R_2\text{-}[[NH(CH_2)_b]_c[NH(CH_2)_d]_e]_f[NH(CH_2)_g]_h\text{-}NHR_2 \qquad (IV)$$

or the formula:

$$R_2\text{-}[X\underset{\displaystyle R_4\text{-}R_5}{\underset{|}{CH}}CO]_i\text{-}R_3 \qquad (V)$$

wherein X is selected from the group consisting of -NH-, -O-, and -S-; R_1 is a substituted or unsubstituted branched or unbranched lower-alkyl chain having from 1 to 4 carbon atoms in the chain; R_2 and R_3 are cross-linking agents covalently linking the polycationic polymer to the oligonucleotide; R_4 is a substituted or unsubstituted branched or unbranched lower-alkyl chain having from 1 to 5 carbon atoms in the chain; R_5 is selected from the group consisting of primary amines, secondary amines, tertiary amines, quaternary ammonium salts, imidazoles, and guanidine groups; R_6 is an alkyl chain having from 1 to 2 carbon atoms; R_7 is selected from the group consisting of hydrogen, branched and unbranched lower-alkyl groups having from 1 to 5 carbon atoms, $-CH_2C_6H_5$, and $-(CH_2)_zCOR_3$, wherein z is an integer ranging from 0 to 3 and R_3 is as defined above; a is an integer ranging from about 3 to about 12; b is an integer ranging from about 2 to about 5; c is an integer ranging from about 0 to about 3; d is an integer ranging from about 2 to about 5; e is an integer ranging from about 0 to about 3; f is an integer ranging from about 2 to about 9; g is an integer ranging from about 2 to about 5; h is an integer ranging from about 0 to about 3; i is an integer ranging from about 3 to about 12; and the ratio of cations in the polycationic polymer to anions in the polyanionic oligonucleotide pair ranges from about 1:8 to about 1.5:1, respectively;

which comprises the steps of:

(1) providing the polyanionic oligonucleotides;

(2) providing the polycationic polymer;

(3) coupling the polyanionic oligonucleotides to the polycationic polymer via the cross linking agents.

16. A paired-ion oligonucleotide comprising a polyanionic oligonucleotide covalently bonded to a polycationic polymer via a cross-linking agent, wherein the polycationic polymer is represented by the formula:

$$R_2\text{-}[XCHCO]_i\text{-}R_3 \qquad (V)$$
$$\quad\;\; |$$
$$\quad R_4\text{-}R_5$$

wherein X is selected from the group consisting of -NH-, -O-, and -S-; R_2 is independently selected from the group consisting of hydrogen, acyl groups, cross-linking agents covalently linking the polycationic polymer to the oligonucleotide, and chemotherapeutic agents; R_3 is selected from the group consisting of -NRR', -OR'', cross-linking agents covalently linking the polycationic polymer to the oligonucleotide, and chemotherapeutic agents; R_4 is a substituted or unsubstituted

branched or unbranched lower-alkyl chain having from 1 to 5 carbon atoms in the chain; R_5 is selected from the group consisting of primary amines, secondary amines, tertiary amines, quaternary ammonium salts, and guanidine groups; R and R' may be the same or different and are independently selected from the group consisting of hydrogen and branched and unbranched lower-alkyl groups, and R'' is selected from the group consisting of branched and unbranched lower-alkyl groups, each lower-alkyl group having from 1 to 6 carbon atoms; wherein at least one of R2 or R3 is a cross-linking agent; i is an integer ranging from about 3 to about 12; and the ratio of cations in the polycationic polymer to anions in the polyanionic oligonucleotide ranges from about 8:10 to about 16:8, respectively.

Figure 1

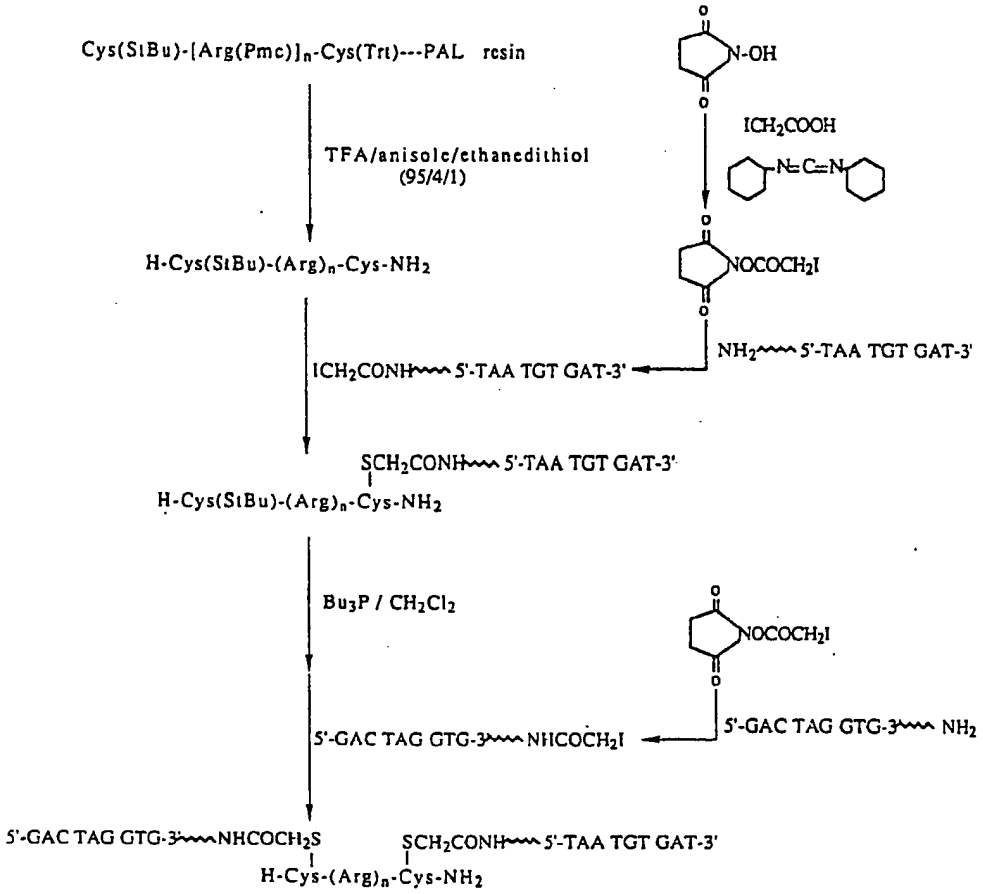


Figure 2

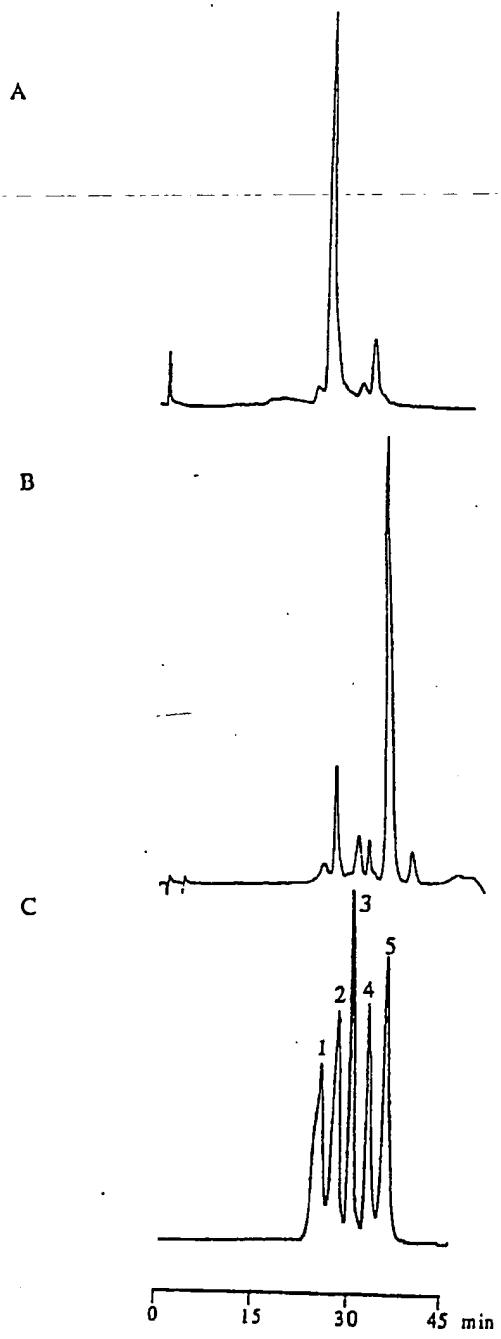


Figure 3

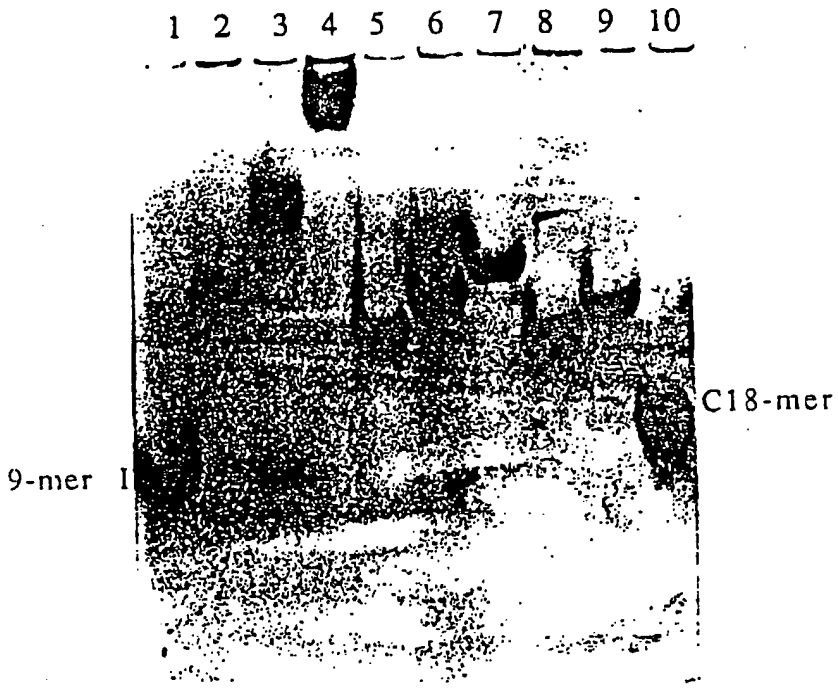


Figure 4

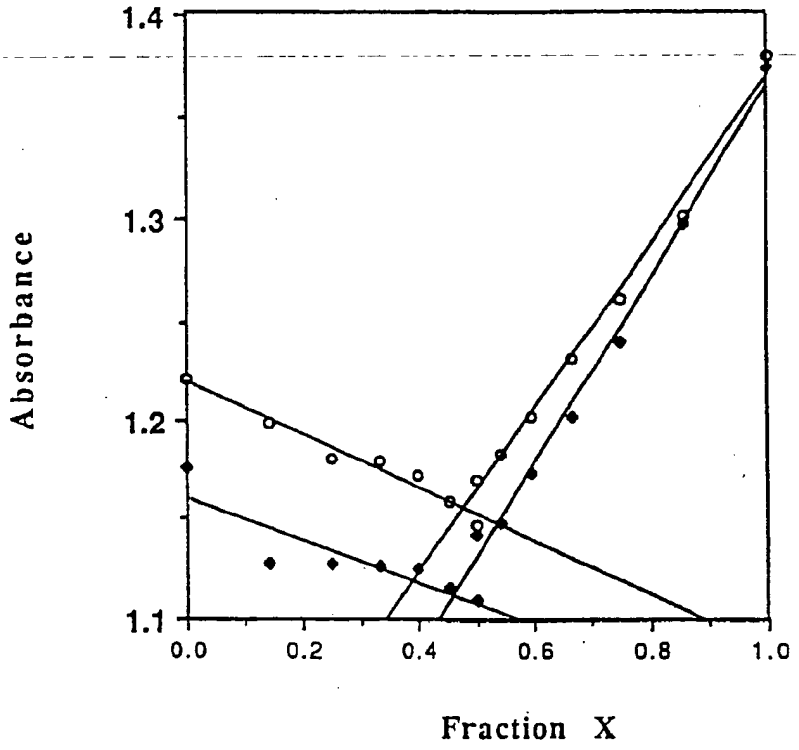


Figure 5

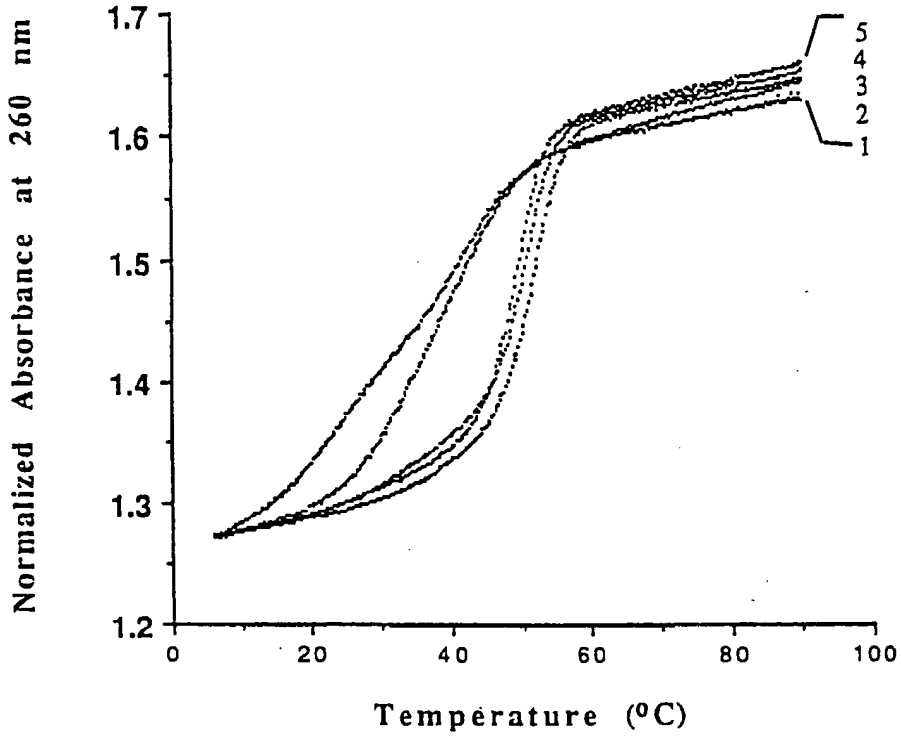

Normalized Absorbance at 260 nm
1.7
1.6
1.5
1.4
1.3
1.2
0
20
40
60
80
100
Temperature (°C)
5
4
3
2
1

Figure 6

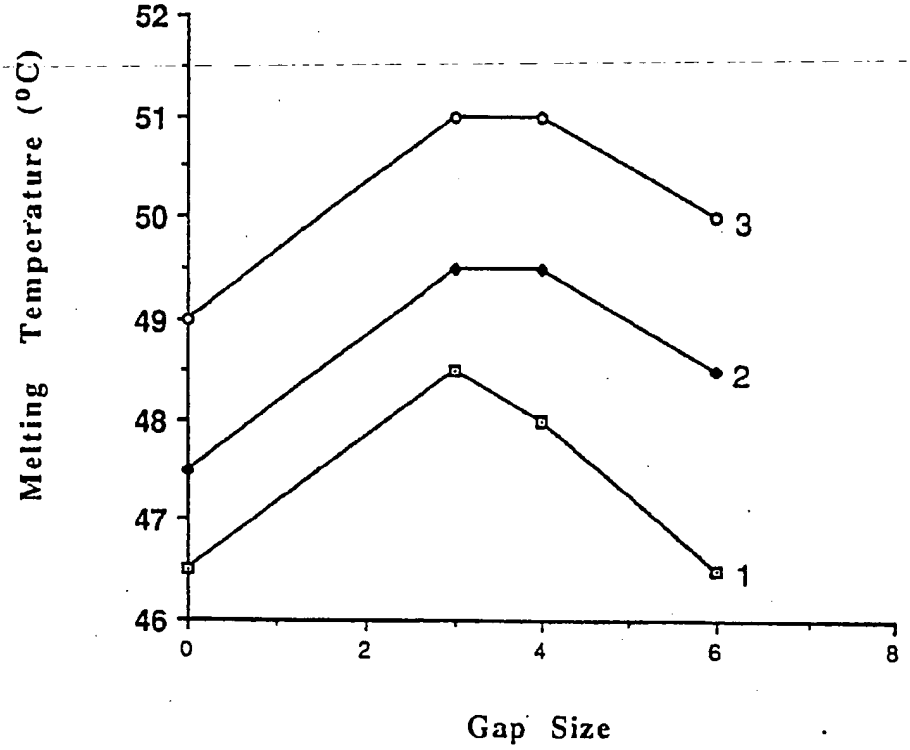
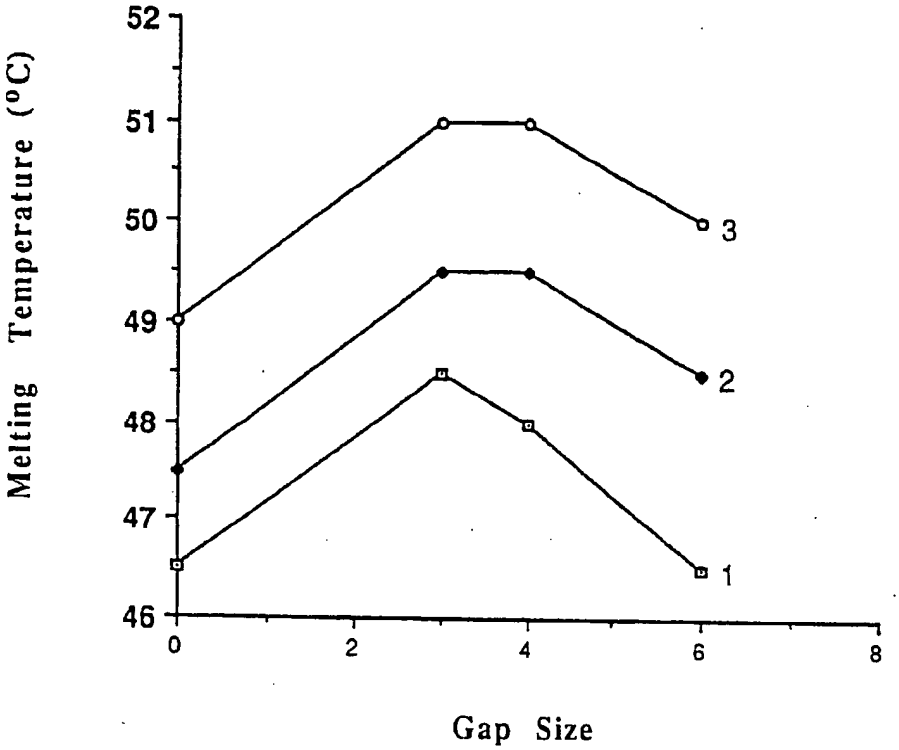


Figure 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09402

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07H 17/00, 23/00; A61K 48/00
US CL :536/25.3, 25.6, 24.1; 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/25.3, 25.6, 24.1; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences USA, Volume 84, issued February 1987, Lemaitre et al, "Specific antiviral activity of a poly(L-lysine)-conjugated oligodeoxyribonucleotide sequence complementary to vesicular stomatitis virus N protein mRNA initiation site", pages 648-652, see entire document.	1-16
Y	Gene, Volume 72, Issued 1988, Leonetti et al, "Antiviral activity of conjugates between poly(L-lysine) and synthetic oligodeoxyribonucleotides", pages 323-332, see entire document.	1-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 NOVEMBER 1994	Date of mailing of the international search report 12 DEC 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEAN SMILEY Jill Warden for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09402

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Bioconjugate Chemistry, Volume 2, Number 6, issued 1991, Tung et al, "Preparation of Oligonucleotide-Peptide Conjugates", pages 464-465, see entire document.	1-16
Y	Proceedings of the American Association for Cancer Research, Volume 34, issued March 1993, Pachuk et al, "Ribozymes targeted to the L6 bcr-abl mRNA for therapy of chronic myelogenous leukemia", page 388, abstract no. 2313, see entire abstract.	1-16
Y	BioTechniques, Volume 12, Number 4, issued April 1992, Borghesi-Nicoletti et al, "Oligonucleotide Hybridization Used to Detect Short Non-contiguous Sequences", pages 474-477, see entire document.	1-16
A	Science, Volume 261, issued 20 August 1993, Stein et al, "Antisense Oligonucleotides as Therapeutic Agents--Is the Bullet Really Magical?", pages 1004-1012, see entire document.	1-16
X --- Y	Tetrahedron, Volume 47, Number 24, issued 1991, Eritja et al, "Synthesis of Defined Peptide-Oligonucleotide Hybrids Containing a Nuclear Transport Signal Sequence", pages 4113-4120, see entire document.	15 --- 1-14, 16

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09402

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09402

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

CAS ONLINE
search terms: structures
CA
search terms: oligonucleotides, deoxynucleotides, DNA, basic, conjugate, bridge, linker, basic, cationic, pseudopeptide-linked, peptide

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-6, 11-12, and 14-15, drawn to a bridged polycationic polymer-oligonucleotide conjugate having formula I, a pharmaceutical composition comprising any claimed conjugate, and a method of using a polycationic polymer as a linker

II. Claims 1-4, 6-12, and 14-15, drawn to a bridged polycationic polymer-oligonucleotide conjugate having formula II, a pharmaceutical composition comprising any claimed conjugate, and a method of using a polycationic polymer as a linker

III. Claims 1-4, 6-12, and 14-15, drawn to a bridged polycationic polymer-oligonucleotide conjugate having formula III, a pharmaceutical composition comprising any claimed conjugate, and a method of using a polycationic polymer as a linker

IV. Claims 1-4, 6, 11-12, and 14-15, drawn to a bridged polycationic polymer-oligonucleotide conjugate having formula IV, a pharmaceutical composition comprising any claimed conjugate, and a method of using a polycationic polymer as a linker

V. Claims 1-4, 6-8, 11-14, and 15-16, drawn to a bridged polycationic polymer-oligonucleotide conjugate having formula V, a pharmaceutical composition comprising any claimed conjugate, and a method of using a polycationic polymer as a linker

and it considers that the International Application does not comply with the requirements of unity of invention (Rules 13.1, 13.2, and 13.3) for the reasons listed below:

Claim 1 comprises 5 distinct compounds in the art. Claims 2-13 further define the compounds of claim 1. Claim 14 comprisesapharmaceutical composition. Claim 15 comprises a method of use of those compounds. Claim 16 represents one to the compounds of claim 1.

The compounds of groups I-V are represented by formulas I-V, respectively. These polymers lack a common significant structural feature. In group I, formula I is a polyamine, polyether, or a polysulfide containing a carbonyl group and an -HN2 as a substituent. In groups II and III, each of formulas II and III contain an NH, O, or S bonded to a substituted alkyl group bonded to an ester, amide or -(C=O)-S group, bonded to a substitued alkyl group, bonded to a carbonyl group. Formulas II and III differ in the placement of the substituted alkyl groups. For formula II, the first substituted alkyl group can contain a benzene ring or a carbonyl group attached to a cross-linking agent. The second substituted alkyl group contains an amine, quaternary ammonium salt, imidazole, or guanidine group. In formula III, the order of the substituted alkyl groups are reversed. Thus, they are placed in a different electronic relationship to the three main functional groups. In group IV, formula IV is a polyamine. In group V, formula V is a polypeptide. A reference anticipating one of these formulats would not render the others obvious.

PCT Rules 13.1 and 13.2 do not provide for multiple distinct products within a single application. See PCT Article 17 (3)(a). The products are not so linked by a special technial featurewithin the meanining of PCT Rule 13.2 so as to form a single inventive concept.